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DENGUE 4 VACCINE DEVELOPMENT

ANNUAL AND FINAL REPORT

by

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September 1, 1987

(For the period 1 April 1984 to 9 December 1986)

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-83-C-3097

University of Hawaii at Manoa
School of Medicine
Honolulu, Hawaii 96816

SECURITY CLASSIFICATION OF THIS PAGE

ADA201953

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: University of Hawaii at Manoa School of Medicine; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): Honolulu, Hawaii 96822	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: Contract No. DAMD17-83-C-3097
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 62770A; PROJECT NO. 3MI-62770A870; TASK NO. AJ; WORK UNIT ACCESSION NO. 027

11. TITLE (Include Security Classification): Dengue 4 Vaccine Development

12. PERSONAL AUTHOR(S): Nyven Marchette, Ph.D.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual and Final	FROM 4/1/84 TO 12/9/86	1987 September 1	33

16. SUPPLEMENTARY NOTATION

17. COSATI CODES		
FIELD	GROUP	SUB-GROUP
06	13	
06	03	

18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
Key words: Virus, arbovirus, dengue, dengue-4, vaccine, attenuation, immunity, RAI (RT)

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

A live attenuated Dengue 4 virus candidate vaccine was prepared by serial passage of wild virus isolates in primary dog kidney cells (PDK) at 32 C. In addition, a candidate challenge wild type virus was prepared from the wild type parent by limited passage at 37 C in primary African green monkey kidney cells (PGMK). These viruses were finally passaged in fetal rhesus lung (FRhL) cells for preparation of Master Seed and Production Seed stocks. An additional passage in FRhL cells to produce a candidate vaccine was done at WRAIR from the Production Seeds. All virus passages and lots of cell cultures were routinely tested for sterility according to a standard protocol. The Production Seeds and Final Product (Candidate vaccine) were safety tested and determined to be free of contamination with bacteria (including mycoplasma), fungi, and adventitious agents according to standard protocols. Biological marker studies were done on Production Seeds. These included: temperature sensitivity, plaque size, ability to produce cytopathic effect (CPE) in LLC-MK2 cells, mouse neurovirulence, monkey viremia, immunogenicity in monkeys (antibody development and

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION	
☐ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS	Unclassified	
22a. NAME OF RESPONSIBLE INDIVIDUAL: Mrs. Virginia Miller	22b. TELEPHONE (Include Area Code): (301) 663-7325	22c. OFFICE SYMBOL: SGRD-RMI-S

19. Abstract (Cont.)

resistance to challenge with virulent wild type parent), and growth in human peripheral blood mononuclear cells (PBMC). The Production Seed (Dengue 4 Carib 341750 PDK-20 FRhL-2) was not temperature sensitive, produces CPE and small to medium sized plaques in LLC-MK2 cells, is neurovirulent for mice, does not produce significant viremia in rhesus monkeys but does stimulate antibody production and confers resistance to challenge with wild virus, and grows well in human PBMC. It is recommended that this candidate vaccine be tested for safety and efficacy in human volunteers.

FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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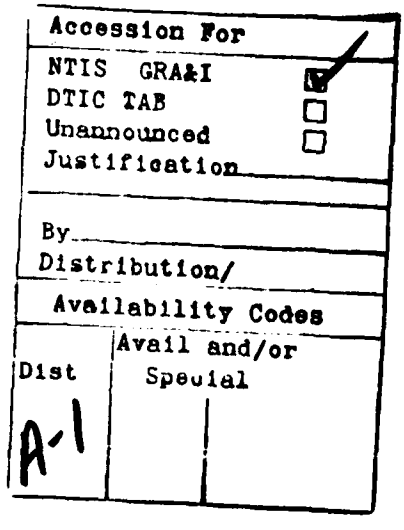

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STATEMENT OF THE PROBLEM

The purpose of the research supported by this contract was to produce one or more live attenuated candidate dengue 4 virus vaccines for testing in human volunteers. Specifically, the work involved:

a) Safety testing of Candidate Dengue 4 (H241) PDK-54 vaccine produced by WRAIR for sterility and for pathogenicity in mice, rabbits, guniea pigs and monkeys.

b) Preparation of a production seed from uncloned Dengue 4 (H241) PDK-35 vaccine isolated from viremic serum of a volunteer vaccinee, and safety test and perform biological marker studies.

c) Select and attenuate a Caribbean Dengue 4 virus strain by serial passage in Primary Dog Kidney (PDK) cells and monitor sterility and affect of passage on biological markers.

d) Prepare a Master Seed and Production Seed of the above attenuated Caribbean Dengue 4 virus strain and safety test and perform biological marker studies.

e) Prepare a Master Seed and Production Seed of the parent wild-type Caribbean Dengue 4 virus strain and safety test and perform biological marker studies.

f) Safety test and perform biological marker studies on Candidate Caribbean Dengue 4 virus Candidate Vaccine prepared at WRAIR from Production Seed described in (d) above.

g) Safety test and perform biological marker studies on Candidate wild type Dengue 4 challenge virus prepared at WRAIR from Production Seed described in (e) above

BACKGROUND

Almost all the most widely used vaccines for prevention of human viral disease are preparations of attenuated live viruses. The yellow fever 17D vaccine is arguably the best vaccine ever produced. Smallpox has been eradicated from the earth by use of the vaccinia virus vaccine. Epidemic poliomyelitis has been essentially eradicated from the United States and other developed countries, principally as a result of mass vaccination with the trivalent Sabin attenuated live virus vaccine. Live attenuated measles, mumps, and rubella vaccines are safe and effective; they have reduced the incidence of these diseases to very low levels in countries where they are widely used. An attenuated live adenovirus vaccine for the prevention of infection with types 4 and 7 is in use by the military. Safe, effective live virus vaccines have been developed to protect domestic animals and pets from infection with rabies, Venezuelan encephalitis, and Rift Valley fever viruses, all of which can also infect and cause disease in humans. All these human and veterinary vaccines produce seroconversion with prolonged antibody production in nearly all recipients and evoke durable protection against disease from infection with wild virus. Their success in controlling viral diseases has been phenomenal.

Despite the considerable efforts being made to develop subunit and synthetic peptide vaccines, work on development of live attenuated virus vaccines continues. Experimental attenuated live virus vaccines

for protection against Japanese encephalitis, Argentinian hemorrhagic fever, rotavirus diarrhea, hepatitis A, influenza, varicella, cytomegalovirus, as well as dengue viruses are in various stages of development.

Attenuation of virulent wild viruses has generally been accomplished simply by serial passage in cultures of cells from non-natural hosts, sometimes at reduced temperature to produce stable temperature sensitive or cold adapted variant strains. Dengue virus types 1 and 2 attenuated for man by serial intracebral passage in infant or weanling mice (1-3) evoked an antibody response in volunteers. A Candidate Dengue 2 virus vaccine developed at WRAIR (4,5) was immunogenic for monkeys (6) and safe for human use (7), but it produced satisfactory immunity only in volunteers immune to yellow fever (7,8). A live attenuated Dengue 2 virus vaccine developed in Thailand has proven to be an effective immmunogen in human volunteers (9).

A live attenuated Dengue 4 candidate vaccine was prepared previously by our laboratory in collaboration with WRAIR (10-13) using the prototype Dengue 4 (H241) strain passaged in primary dog kidney (PDK) cells. This vaccine was cloned by limit dilution, a candidate vaccine prepared in fetal rhesus lung (FRhL) cells, and tested for safety and immunogenicity in human volunteers. It was over-attenuated for humans and did not produce satisfactory immunity to dengue infection (14). On the basis of these results, the Ad Hoc Scientific Review Committee for Dengue Vaccines decided that renewed efforts should be made to develop a new live attenuated Dengue 4 virus vaccine.

APPROACH TO THE PROBLEM

Three approaches were used:

1) Preparation of a new candidate vaccine from the uncloned Dengue 4 (H241) PDK-52 Mastere Seed previously made and safety tested in our laboratory.

2) Preparation of a new vaccine beginning with a virus recovered from the viremic plasma of one of the volunteers vaccinated with the cloned Dengue 4 Candidate Vaccine described above. For this purpose, a sample of viremic plasma from this volunteer was sent to us for passage in PDK cells and preparation of Master Seed and Production Seed from which a new Candidate Vaccine would be prepared for testing in human volunteers.

3) Preparation of a candidate vaccine from a more recent Dengue 4 virus isolate from human patients in the Caribbean. Consequently, viruses in viremic plasma from several dengue patients in Colombia and

Dominican Republic were recovered in T. splendens mosquitoes and passaged in PDK cells and in Primary African Green Monkey Kidney (PGMK) cells according to the original protocol.

RESULTS

Uncloned Dengue 4 (H-241) PDK52.

The uncloned Dengue 4 (H-241) PDK-52 virus was passaged two more times to produce a new PDK53 Master Seed and a PDK54 Production Seed. The production seed was safety tested and shown to be free of bacterial, fungal, and adventitious agents. Biological marker studies demonstrated that the seed contains mostly virus that forms pinpoint plaques in standard plaque assay in LLC-MK2 cells. It is , however, a mixed population with a small proportion of viruses forming medium sized plaques. The virus is temperature sensitive, does not produce CPE in LLC-MK2 cells, has reduced mouse neurovirulence, grows in human PBL, produces viremia in 50% of rhesus monkeys tested, and humoral immune response in 75%. All monkeys with an antibody response resisted challenge with the wild parent strain (Table 1). Approximately half the 1000 ml of Production Seed was sent to WRAIR for preparation of a candidate vaccine.

Cloned Dengue 4 (H-241) PDK35 TD3 Human isolate.

The cloned Dengue 4 (H-241) PDK35 TD3 candidate vaccine isolate from viremic plasma from volunteer S.G. was serially passaged in PDK cells at 37 C and 32 C. The virus quickly lost viability when passed at 37 C, but gradually adapted to PDK cells when cultured at 32C (Table 2). An attempt to prepare a Master Seed in FRhL cells from the fifth PDK passage was not successful even when virus was harvested after $MgSO_4$ treatment (Table 3). The second passage in FRhL for a production seed failed to replicate (Table 3). An attempt to produce a Master Seed in human diploid lung fibroblasts (MRC-5) also was not successful (Table 4).

The virus replicated in PGMK cells, but not in C6/36 cells (Table 5).

Considering the difficulty encountered in readapting this virus to cell substrates suitable for human use, it was decided to abandon further attempts to prepare a candidate vaccine with this strain.

Caribbean Dengue 4 Colombia (341750).

The Caribbean Dengue 4 virus Colombia 341750 was selected for vaccine development on the basis of its ability to replicate in PDK cells and was serially passaged in these cells at 32 C. It adapted rapidly to this substrate and the plaque size decreased from uniformly large (>6mm) to mostly small (2-4 mm) although there were usually a few medium sized (4-6 mm) plaque forming viruses in the PDK passages (Table 6). Between passage PDK-23 and PDK-36, the plaque size became

increasingly heterogeneous.

Biological markers on PDK-15 and PDK-30 were remarkably similar to the wild-type parent except in plaque size and in production of viremia in monkeys. Although neither PDK-15 nor PDK-30 produced viremia, the monkeys were protected from challenge with wild parent virus (Table 7).

After consultation with colleagues at WRAIR, PDK-20 was selected for passage into FRhL cells for production of a Master Seed. The pooled 1000 ml Master Seed had a titer of 5.1×10^5 pfu/ml. It was tested for sterility, and a 1000 ml Production Seed was prepared by a second passage in FRhL cells. The Production Seed was shown to be free of bacterial, fungal and adventitious agents. Approximately half the Production Seed was sent to WRAIR for preparation of the Candidate Vaccine.

A sample of the Candidate Vaccine, Dengue 4 (341750) PDK20 FRhL4, was safety tested and shown to be free of bacterial, fungal, and adventitious agents (Appendix).

The identity of the Production Seed virus as a strain of Dengue 4 was confirmed by plaque reduction neutralization tests (PRNT) using a Dengue 4 immune mouse ascitic fluid from mice immunized with Dengue 4 (H-241) in this laboratory. The Candidate vaccine virus was identified as Dengue 4 using a lyophilized Dengue 4 immune mouse ascitic fluid from mice immunized with Dengue 4 (H-241) prepared by Dr. Robert Shope, Yale University; and an immune serum from a monkey inoculated with the parent Dengue 4 challenge virus in this laboratory. The preparation of the vaccine, safety tests, and identity confirmation data are outlined in the Appendix.

A Production Seed of the parent Caribbean Dengue 4 (341750) virus for use in challenging vaccinated monkeys and human volunteers was prepared by serially passing the first mosquito passage five times in PGMK cells and twice in FRhL cells. The Production Seed virus maintained its parental large plaque forming phenotype, produced viremia in monkeys and was immunogenic. Approximately 500 ml of the Production Seed was sent to WRAIR for preparation of a Candidate Challenge Virus.

Virus Replication in Human Peripheral Blood Mononuclear Cells as a Virulence Marker.

An attempt was made to evaluate the reliability of Dengue 4 virus replication in human peripheral blood mononuclear cells (PBMC) as a virulence marker. Four of five wild (presumably virulent) Dengue 4 virus isolates from cases of dengue fever or dengue hemorrhagic fever grew poorly in PBMC. The fifth isolate grew to high titer (Table 8), but it had been grown only in mosquito cells; the other strains had

been passaged several times in rhesus monkey kidney (LLC-MK2) cells.

Serial passage of Dengue 4 (H-241) and Dengue 4 (341750) strains in permissive LLC-MK2 cells at 32 C did not significantly alter their ability to replicate in PBMC. Five serial passages in PGMK cells and two serial passages in FRhL cells at 37 C did not significantly alter the ability of wild type Dengue 4 (341750) to replicate in PBMC compared to virus serially passed in LLC-MK2 cells. Passage in C6/36 mosquito cells at 32 had no significant effect on replication of either H-241 or 341750 strains in PBMC (Table 9).

Passage of Dengue 4 H-241 virus in PDK at 32 C altered its ability to replicate in PBMC. The 17th and 32nd PDK cell passages replicated to significantly higher titer in PBMC than the parent wild type LLC-MK2 passaged virus. The 54th PDK passage, however, grew very poorly and virus production was not significantly greater than that of the parent virus (Table 10).

Parental Dengue 4 341750 virus also grew poorly in PBMC. By the sixth PDK passage, a population had been selected that grew to significantly higher titer in PBMC that the parent virus. This ability to replicate in these cells was maintained through PDK-30. One passage of PDK-20 in FHrL cells, however, resulted in decreased growth, which was almost fully restored by a second FRhL passage (Table 11).

The ability of Dengue 4 H-241 virus to replicate in PBMC was independent of the other biological markers tested (Table 1). However, replication of Dengue 4 341750 in PBMC was correlated with small plaque size and inability to produce viremia in rhesus monkeys (Table 12).

DISCUSSION

The terms of the contract have been fulfilled with the preparation and testing of potential live attenuated Dengue 4 virus vaccines. The various seed stocks have been deposited at WRAIR with portions of each continuing to be maintained in the Tropical Medicine Laboratory.

A Production Seed of the uncloned Dengue 4 (H-241) PDK54 FRhL2 virus was prepared and shown to be of sufficient titer for use as a vaccine and to be free of bacterial, fungal, and adventitious agents.. A decision to proceed with the production of a Candidate Vaccine at WRAIR had not been made at the time of the termination of this contract.

Development of a suitable product from the Dengue 4 (H-241) PDK35 TD3 S.G. viremic plasma was unsuccessful because of the inability of the virus to replicate in cell substrates suitable for use in humans. It replicates to low titer in PDK cells at 32 C, but does not replicate in FRhL cells or human diploid fibroblasts (MRC-5). There was some

indication that repeated serial passage in PDK cells might select for a population sufficiently adapted to this substrate. However, after 12 passages, the plaque phenotype began to change from uniformly medium plague forming to mixed populations of pinpoint and medium sized plaque forming viruses. Further work with this strain was suspended to concentrate on development of the Dengue 4 Caribbean 341750 vaccine.

The plaque characteristics and monkey virulence and immunogicity data of the 15h and 30th PDK passages of Dengue 4 (341750) virus suggested that it had become attenuated at the 15th passage level. The data were reviewed by us and colleagues at WRAIR, and the decision was made to prepare the appropriate FRhL virus seed stocks from the PDK20 passage. A final product Candidate Vaccine (Dengue 4 341750 PDK20 FRhL4) is of small plaque phenotype, is immunogenic for rhesus monkeys without producing detectable viremia and grows well in human peripheral blood mononuclear cells in vitro.

RECOMMENDATIONS

The Dengue 4 PDK-20 FRhL-4 Candidate Vaccine is free of detectable bacterial, fungal and adventitious agents, and it has in vitro and in vivo (monkeys) characteristics of modified virulence. It is recommended that safety and immunogenicity trials in human volunteers be scheduled.

If this preparation proves to be a safe and effective immunogen, experiments should be undertaken to determine the location and characteristics of the lesion(s) responsible for the attenuation phenotype.

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Table 1. Dengue 4 (H-241) PDK-54 (Production Seed) Biological Markers

Marker	D4 (H-241) Parent	D4 (H-241) PDK-54
Temperature sensitivity	no	yes (cut off 39)
Plaque size	large	pinpoint
CPE in LLC-MK2 cells	yes	no
Growth in human PBMC	no	yes
Mouse neurovirulence (mean day of death) in 1-day old mice	11.0	13.7
Rhesus monkey virulence		
Viremia	14/14	2/4
Hemagglutination inhibition antibody	14/14	3/4
Neutralizing antibody	14/14	3/4
Challenge resistance of vaccinated monkeys to wild parent virus		
Viremia	ND*	1/4
Hemagglutination inhibition antibody	ND	3/3
Neutralizing antibody	ND	3/3
Safety test for bacteria, fungi, mycoplasma	ND	negative
Tests for adventitious agents in:		
Animals (mice, guinea pigs, rabbits)	ND	negative
Tissue culture (PMK, PRK, WI-38, PGMK)	ND	negative
Breakthrough neutralization test	ND	satisfactory

*Not done

Table 2. Passage of Dengue 4 (H-241) PDK-35 TD3 S.G. viremia isolate in PDK cells.

Passage	pfu/ml 32 C	pfu/ml 37 C	Plaque size
PDK-1	2.3×10^2	1.0×10^3	MP*
2	3.3×10^2	15	MP
3	3	0	MP
4	42	0	MP
5	4.3×10^3		MP
6	6.0×10^3		MP
7	3.0×10^3		MP
8	1.6×10^3		MP
9	1.3×10^2		MP
10	2.3×10^2		MP
11	8.0×10^2		MP
12	2.5×10^3		PP and MP
13	2.5×10^3		PP and MP

* Diameter of large plaque (LP) = >6mm; medium plaque (MP) - 4-6 mm; small plaque (SP) = 2-4 mm; pinpoint plaque (PP) = <2 mm.

Table 3. Preparation of Dengue 4 (H-241) PDK-35 TD3 S.G. viremia master seed in FRhL cells

Lot No.	Day of Harvest	$MgSO_4$	Titer (Pfu/ml)	Plaque size
1*	7	no	0	---
2	7	yes	0	---
3	8	no	15	MP**
4	8	yes	12	MP
5	9	no	2.5×10^2	MP
6	9	yes	33	MP
7	10	no	3.0×10^2	MP
8	10	yes	43	MP
9	7	no	0	---
10	7	yes	0	---
11	8	no	10	MP
12	8	yes	0	---
13	9	no	17	MP
14	9	yes	13	MP
15	10	no	33	MP
16	10	yes	20	MP

*Volume of each lot - 100 ml.
**See footnote Table 2.

Table 4. Preparation of Dengue 4 (H-241) PDK-35 TD3 S.G. viremia in MRC-5 cells

Lot No.	Day of Harvest	$MgSO_4$	Titer (pfu/ml)	Plaque size
1*	7	no	0	---
2	7	yes	0	---
3	8	no	12	MP**
4	8	yes	0	---
5	9	no	13	MP/LP
6	9	yes	0	---
7	10	no	48	MP/LP
8	10	yes	5	MP/LP
9	11	no	45	MP/LP
10	11	yes	5	MP/LP
11	12	no	71	MP/LP
12	12	yes	12	MP/LP
13	13	no	40	Mp/LP
14	13	yes	8	MP/LP
15	14	no	30	MP/LP
16	14	yes	7	MP/LP

*Volume of each lot - 100 ml.
**See footnote Table 2

Table 5. Passage of Dengue 4 (H-241) PDK-35 S.G. viremia in PGMK cells and C6/36 cells

	PGMK cells 37 C		C6/36 cells 32 C
Passage	Titer (pfu/ml)	Plaque size	Titer (pfu/ml)
1	1.5×10^2	MP*	0
2	6.8×10^2	MP	0
3	10	MP	0
4	1.3×10^3	MP	0
5	1.0×10^4	MP	0

*See footnote Table 2.

Table 6. Plaque assays of serial passages of Dengue 4 Carib (341750) M-1 in PDK cells and of FRhL passages of PDK-20.

Passage	Titer	Plaque size	Passage	Titer	Plaque size
PDK-1	3.3*	LP**	PDK-21	5.1	SP
2	3.7	SP	22	6.2	SP
3	3.4	SP	23	5.3	SP/PP
4	4.1	SP	24	5.3	SP
5	4.2	SP/MP	25	5.4	SP/MP
6	4.9	SP	26	5.8	SP/MP
7	4.1	SP	27	5.5	SP/PP
8	5.1	SP	28	4.6	SP/MP
9	4.9	SP	29	6.2	SP/MP
10	5.1	SP/MP	30	5.4	SP/PP
11	4.7	SP	31	4.5	SP/PP/MP
12	4.8	SP	32	5.0	SP
13	4.8	SP	33	4.8	SP/MP
14	4.4	SP/MP	34	4.7	SP/MP
15	4.6	SP	35	5.2	SP/MP
16	5.7	SP	36	5.4	SP/MP/LP
17	5.0	SP	PDK-20 FRhL-1	5.7	SP/MP
18	4.9	SP/MP	2	5.0	SP/MP
19	6.2	SP	3	5.5	SP/MP
20	5.4	SP	4	5.2	SP/MP

* Log_{10} pfu/ml; ** LP-large plaque >6 mm diameter, MP-medium plaque 4-6 mm diameter, SP-small plaque 2-4 mm diameter, PP-pinpoint <2 mm diameter.

Table 7. Dengue 4 (341750) PDK-15 and Dengue 4 (341750) PDK-30 biological markers

	D4 (341750) Parent	D4 (341750) PDK-15	D4 (341750) PDK-30
Temperature sensitiviey	no	no	no
Plaque size	large	small	small/pinpoint
CPE in LLC-MK2 cells	yes	yes	reduced
Growth in human PBMC	+/-	+	+
Mouse neurovirulence (mead day of death) in 1-day old mice	10.5	10.5	11.5
Rhesus monkey virulence			
Viremia	4/4	0/2	0/2
HI antibody	4/4	2/2	1/2
Neutralizing antibody	4/4	2/2	0/2
Challenge of vaccinated monkeys with wild parent virus			
Viremia	0/4	0/2	0/2
HI antibody	4/4	2/2	2/2
Neutralizing antibody	4/4	2/2	2/2

Table 8. Replication of wild DEN 4 virus strains in human PBMC.

Virus	$\bar{x}$*	SD	p
H-241 MK_2-3	1.4	0.8	--
341750 MK_2-1	1.6	0.9	NS**
1036 MK_2-1	1.8	1.1	NS
4328S MK_2-6 C6/36-3	1.5	0.6	NS
814669 TRA-1	4.2	0.8	<0.001

* Mean Log_{10} pfu/ml days 3-5 of culture of at least 3 separate experiments. SD = standard deviation.

** Not significantly different from H-241 MK-3.

Table 9. Effect of virus passage, cell substrate, and temperature on replication of Dengue 4 (H-241) and Dengue 4 (341750) strains in PBMC.

	DEN-4-H241			DEN-4-341750		
	$\bar{x}$	SD	p	$\bar{x}$	SD	p
Passage in LLC-MK2 cells						
32°C	0.89*	0.49	>0.05	1.01	0.39	>0.05
37°C	1.45	0.79		1.62	0.85	
Passage in						
PGMK**	ND+	ND	ND	0.60	--	>0.05
LLC-MK_2				1.62	0.85	
Passage in						
FRhL	ND	ND	ND	2.50	0.19	>0.05
LLC-MK_2				1.62	0.85	
Passage in						
C6/36	1.52	1.31	>0.05	2.83	0.77	>0.05
LLC-MK_2	1.24	0.86		1.62	0.85	

* Mean Log_{10} pfu/ml days 3-5 of culture of at least three separate experiments.
** See footnote Table 1 for cell culture abbreviations.
+ ND = Not done.

Table 10. Replication of PDK passaged Dengue 4 (H-241) in human PBMC.

Passage	x	SD	p
Parent*	1.24**	0.73	--
PDK-17	3.51	0.40	<0.01
PDK-32	3.11	0.62	<0.01
PDK-54	1.43	0.65	>0.05
SG PDK-6	1.26	0.71	>0.05

* Stock laboratory virus prepared in LLC-MK_2 cells..
** Mean Log_{10} pfu/ml days 3-5 of culture of at least three separate experiments. SD = standard deviation.

Table 11. Replication of PDK passaged Dengue 4 ((341750) virus in human PBMC.

Passage	Virus Stock Titer	$\bar{x}$	SD	p
LLC-MK_2-1-C6/36-1 (Parent)	6.3*	2.8**	0.8	--
PDK-1	3.3	ND***		
PDK-1-C6/36-1	6.2	2.3	0.9	>0.05+
PDK-2	3.7	ND		
PDK-2-C6/36-1	6.2	2.8	0.8	>0.05
PDK-3	3.4	ND		
PDK-3-C6/36-1	6.4	3.7	1.0	>0.05
PDK-4	4.1	ND		
PDK-4-C6/36-1	6.8	3.8	1.0	>0.05
LLC-MK_2-1 (Parent)	5.5	1.6	0.9	--
PDK-6	4.8	4.1	0.6	<0.01
PDK-10	5.2	3.7	0.5	<0.05
PDK-15	4.6	4.1	1.2	<0.01
PDK-20	5.0	4.5	0.7	<0.01
PDK-30	5.4	4.0	0.9	<0.01
PDK-20	5.0	4.5	0.7	--
PDK-20-FRhL-1	5.6	1.6	0.8	<0.01
PDK-20-FRhL-2	4.9	3.8	0.6	>0.05

* Mean Log_{10} pfu/ml in LLC-MK2 or BHK cells.

** Mean Log_{10} pfu/ml days 3-5 of PBMC culture of at least three separate experiments. SD = standard deviation.

***Not done.

+ PDK 1-4-C6/36-1 passages were compared to parent LLC-MK_2-C6/36-1 passage; PDK 6-30 were compared to parent LLC-MK_2-1. There was no statistically significant difference between the parent C6/36-1 passage and the LLC-MK_2-1 passage from which it was derived. PDK-20 FRhL-1 and 2 were compared to PDK-20.

Table 12. Biological markers of DEN 4-341750 experimental vaccine strain.

Marker	Wild Parent	PDK 6	PDK 10	PDK 15	PDK 20	PDK 30	PDK FRhL-1	PDK FRhL-2
ts 39°C	no	ND##	ND	no	no	no	no	no
CPE in MK_2	yes	ND	ND	yes	ND	yes	ND	yes
Plaque size	LP*	SP	MP,SP	SP	SP	PP,SP	SP	MP,SP
Mouse neurovirulence	10.0**	ND	ND	10.5	ND	11.5	ND	9.0
Monkey viremia	4/6***	ND	ND	0/2	ND	0/2	ND	1/4###
Antibody titer HAI (day 42)	160-1280	ND	ND	20	ND	10-20	ND	<10-10
Antibody Titer PRNT+ (day 42)	1366	ND	ND	29	ND	<10	ND	66
Resistant to challenge with parent virus	yes++	ND	ND	yes	ND	yes	ND	yes
Growth in Human PBMC	1.6#	4.1	3.7	4.1	4.5	4.0	1.6	3.8

See footnotes from Table 6.
##Not done
###One monkey had viremia (13 pfu/ml) on one day only. Wild type virus viremia generally lasted 3-6 days and was of higher titer.

APPENDIX

Dengue 4 Caribbean (341750) Candidate Vaccine Preparation

VIRUS STRAIN

Dengue virus type 4 (DEN-4) strain 341750 Carib.

PASSAGE HISTORY

Viremic human plasma was passed one time in adult Toxorhynchites amboinensis mosquitoes by intrathoracic inoculation; 20 times in primary dog kidney (PDK) cells; and four times in diploid fetal rhesus lung cells (FRhL). The master seed was the first passage in FRhL cells; the production seed was the third FRhL passage. The vaccine was prepared by passage of the production seed in FRhL cells at passage 16

IDENTITY.

The human plasma isolate was serotyped by plaque reduction neutralization tests (PRNT) as DEN 4. The production seed virus (FRhL-3) lot 3 was identified as DEN 4 by neutralization tests using a DEN 4 immune mouse ascitic fluid from mice immunized with DEN-4 (H-241) 10-29-70 by the Department of Tropical Medicine laboratory. Candidate vaccine (FRhL-4) lot 2 was identified as DEN-4 using a lyophilized DEN-4 immune mouse ascitic fluid from mice immunized with DEN-4 (H-241) 1-24-66 prepared by Dr. Robert Shope, Yale University; and by using immune serum from a monkey inoculated with the parent DEN-4 (341750) challenge virus.

PRODUCTION SEED SAFETY TESTS (UNCLARIFIED FLUID POOL)

Microbial sterility (control fluids)

Sterility in thioglycollate and trypticase soy broth as specified by 21 CFR 610.12 was Satisfactory.

Cell cultures	Neut.DEN-4 harvest	Un-neut. DEN-4 incub. at 40.5 C	Control Fluids d4, d14	Results
Primary African green monkey kidney	10 ml	10 ml	10 ml	Sat.
Subcultured prim Afr green monk kidney	10 ml	10 ml	10 ml	Sat.
Primary rhesus monkey kidney	10 ml	10 ml	10 ml	Sat.
Fetal rhesus lung	10 ml	10 ml	10 ml	Sat.
WI-38	10 ml	10 ml	10 ml	Sat.

Animals	No. Inoculated Neut DEN-4 harvest	Un-neut DEN-4 harvest	Control fluid	Results
Adult mice	-	20	-	Sat.
Suckling mice	8 litters	-	3 litters	Sat.
Suckling mice Subpassage	4 litters			Sat.
Guinea pigs	-	2	2	Sat.
Rabbits	-	2	2	Sat.

Microbial sterility.

Thyoglycollate broth (as specified by 21 CFR 610.12).
Satisfactory.
Trypticase soy broth (as specified by 21 CFR 610.12).
Satisfactory.
Mycoplasma broth and agar (21 CFR 610.12); Hoescht stain.
Satisfactory.

Reverse transcriptase assay.

Production seed and control fluids from FRhL cells. No evidence of retroviral activity.

VACCINE SAFETY TESTS

Microbial sterility (control fluids)

Sterility in thioglycollate and trypticase soy broth as specified in 21 CFR 610.12. Satisfactory

Cell Culture	Neut. DEN-4 Harvest	Pre-inoc Fluids	Control Fluids (day 4-6)	Control Fluids (day 14)	Results
Primary African green monkey kidney	10 ml	10 ml	10 ml	10 ml	Sat.
Subculture PGMK	10 ml	10 ml	10 ml	10 ml	Sat.
Primary Rhesus monkey kidney	10 ml	10 ml	10 ml	10 ml	Sat.
Fetal rhesus Lung	10 ml	10 ml	10 ml	10 ml	Sat.
WI-38	10 ml	10 ml	10 ml	10 ml	Sat.

Number Inoculated

Animals	DEN-4 Harvest (not neut.)	DEN-4 Neut.	Results
Adult mice	32	ND	Satisfactory
Suckling mice	ND	56	Satisfactory
Subpassage Suckling mice	ND	32	Satisfactory

Microbial sterility

Thioglycollate broth (as specified by 21 CFR 610.12)-Final product. Satisfactory

Trypticase soy broth (as specified by 21 CFR 610.12)-Final product. Satisfactory.

Mycoplasma broth and agar (as specified by 21 CFR610.12), and Hoescht stain-Unclarified vaccine, control fluid day 6, control fluid (Day 14). Satisfactory

Final container safety tests

	No. Inoculated	Results
mice	2	Satisfactory
Microbial sterility as specified by 21 CFR 610.12		Satisfactory

Identity (by plaque reduction neutralization assay in LLC-MK2 cell culture monolayers). Satisfactory

DETAILED SUMMARY OF STUDIES

Virus Passage History

Viremic plasma containing DEN-4 strain 341750 Carib virus was passed one time in Toxorhynchites amboinensis mosquitoes by intrathoracic inoculation and then serially in primary dog kidney cells at seven (7) day intervals for 35 passages at 32 C. Undiluted cell culture supernatant fluids from infected flasks were used as inocula for each subsequent passage. A small pool of seed virus was prepared of passage levels 5, 10, 15, 20, 25, 30, and 35. The 20th passage level virus (PDK-20) was passed in diploid fetal rhesus lung (FRhL) cells to form a master seed (PDK-20 FRhL-1), which was passed again in FRhL cells to form a large pool of the production seed (PDK-20 FRhL-2). The production seed was sent to WRAIR where a further passage in FRhL was made and the candidate vaccine (PDK-20 FRhL-4) was prepared it.

A detailed summary of the passage history of DEN-4 strain 341750 Carib is provided in Table 1.

Table 1. Passage history of DEN-4 341750 Carib PDK-20-FRHL-4

Cell culture passage No.	Cell lot No.	Date inoculated	day of harvest	Comments
24	DBS FRhL (WRAIR)			Vaccine
23	DBS FRhL (WRAIR)			Prod. Seed
22	DBS FRhL P-16 Lot 24	23 Oct. 84	3-7	Prod.
		2 Nov. 84	3-7	Seed
		1 Feb. 85	3-7	5 lots
		4 Feb. 85	3-7	
		8 Mar. 85	3-7	
21	DBS FRhL P-16 Lot 24	22 Aug. 84	5-10	Master
		24 Aug. 84	5-10	Seed
20	PDK 152 sublot 11	12 Dec. 83	7	
19	PDK 152 sublot 10	9 Dec. 83	7	
18	PDK 152 sublot 9	11 Nov. 83	7	
17	PDK 152 sublot 9	4 Nov. 83	7	
16	PDK 152 sublot 9	28 Oct. 83	7	
15	PDK 152 sublot 8	21 Oct. 83	7	
14	PDK 152 sublot 8	14 Oct. 83	7	
13	PDK 152 sublot 7	7 Oct. 83	7	
12	PDK 152 sublot 7	30 Sept. 83	7	
11	PDK 152 sublot 7	23 Sept. 83	7	
10	PDK 152 sublot 6	16 Sept. 83	7	
9	PDK 152 sublot 6	9 Sept. 83	7	
8	PDK 152 sublot 6	2 Sept. 83	7	
7	PDK 152 sublot 6	26 Aug. 83	7	
6	PDK 152 sublot 5	18 Aug. 83	7	
5	PDK 152 sublot 5	11 Aug. 83	7	
4	PDK 152 sublot 5	3 Aug. 83	7	
3	PDK 152 sublot 4	21 July 83	7	
2	PDK 158 sublot 3	2 June 83	7	
1	PDK 158 sublot 3	26 May 83	7	
Mosq. 1	T. amboinensis adults (No. 605351)	10 May 83	7	
Human viremic plasma				

Tests on PDK-20 FRhL-2 production seed

Tests for bacterial and mycoplasmal contaminants were negative after culturing the seed virus in appropriate media. Tests for adventitious agents were performed in adult and suckling mice, rabbits, guinea pigs and four (4) types of cell culture. All tests for adventitious agents were negative.

PREPARATION OF AN ATTENUATED LIVE DEN-4 VACCINE

Facilities and Personnel

All work on this vaccine except for the final production of candidate vaccine was performed in the Department of Tropical Medicine and Medical Microbiology, Atherton Building, Leahi Hospital, Honolulu, HI. An isolated laboratory was used exclusively for this project. All virus and cell culture work was done in a laminar flow hood in this laboratory. This room and the hood were decontaminated each day before initiation of virus passage work. Only dengue virus and cell cultures suitable for vaccine production were handled in this area. Supplies of cell culture media and other reagents used in the vaccine production were stored in this room. A separate liquid nitrogen refrigerator, used exclusively for storage of cells, and a -80 C freezer, used for virus storage were in a room adjacent to the laboratory. Access to the area was restricted to the technician assigned to the project and to the director of the study. Clean laboratory garments reserved for this room were worn nowhere else.

Titration of the various passage level pools and biological marker studies on the virus were done in laboratory rooms in the Department of Tropical Medicine located in a separate but adjacent building. Each laboratory is equipped with laminar flow virus safety hoods. Animal studies were conducted in separate mouse, guinea pig, rabbit, and rhesus monkey rooms in the department's animal facility.

Tests performed on vaccine and control fluids prior to clarification

Safety test samples (20 ml) were removed from pooled bulk DEN-4 vaccine prior to clarification and from pooled control fluids. Each sample was tested for microbial sterility in thioglycollate and trypticase soy broth; and for mycoplasma contamination in mycoplasma broth and agar aerobically and anaerobically and by Hoescht stain. All tests were satisfactory. A total of 20 ml of bulk unclarified vaccine was neutralized with DEN-4 specific mouse ascitic fluid and inoculated into flasks of primary monkey kidney (PMK) cells, primary African green monkey kidney (PGMK) cells, fetal rhesus lung (FRhL) cells, and WI-38 diploid human lung fibroblasts. A similar quantity of pooled control fluid was inoculated into the same types of cells. These cultures were observed for morphologic or cytopathic changes for 14 days. The supernatant fluids from PGMK flasks were subpassed into additional flasks of PGMK cells which were observed for an additional 14 days. No evidence of cytopathic agents or bacterial contamination was observed and the tests were judged to be satisfactory.

Animal inoculation

Rabbits: Two New Zealand white rabbits, weighing 1.4-1.8 kilograms, were each inoculated with DEN-4 unclarified virus subcutaneously with 9.0 ml and intradermally in the subcapular area with 1.0 ml given in 4 sites, 0.25 ml at each site. The same number of rabbits received control fluid for DEN-4.

All the rabbits remained healthy throughout the 21 day test period. No lesions were observed at the sites of inoculation and there was no evidence of B-virus or other viral infection.

Adult mice: Thirty two adult mice (15-20 g) were inoculated intraperitoneally with 0.5 ml and intracerebrally with 0.03 ml of unclarified vaccine. All survived the 21 day test period. None of the mice showed signs of LCM virus infection or other signs of disease.

Suckling mice: Two groups of suckling mice less than 24 hours old were inoculated intraperitoneally with 0.1 ml and intracerebrally with 0.01 ml of either DEN-4 neutralized unclarified vaccine or control fluid. At the end of 14 days, a 20 per cent brain homogenate pool was made from the surviving suckling mice and used to inoculate 32 additional suckling mice using supernatnat fluid of a 1:5 dilution of the brain suspension. Mice that did not survive the test for 21 days were shown to have died of dengue virus infection by subculture and identification of the virus as DEN-4 by immunofluorescence assay indicating that the DEN-4 antiserum (DEN-4 strain H-241 hyperimmune mouse ascitic fluid) used in the test incompletely neutralized the undiluted heterologous strain..

Guinea pigs: Two male guinea pigs, 200-250 g. were inoculated intraperitoneally with 5.0 ml and intracerebrally with 0.1 ml of virus or control fluids. The animals were observed daily for 42 days. The daily rectal temperature of each guinea pig was recorded for the final 21 days of the holding period. There were no observable signs of illness and no significant elevation of temperature in any of the animals.

3.1.3 Tests for mycoplasma (21 CFR 610.30). The glucose and arginine media used for inoculation of vaccine and control fluids are described in the Appendix. Procedures for testing followed 21 CFR 610.30 with Mycoplasma pneumoniae, M. arthrididis, and M. hominis serving as positive controls. Ten agar plates of each medium were inoculated with a total of 2.0 ml of vaccine or control fluids and 4 broth tubes of each medium were inoculated with a total of 1.0 ml of vaccine or control fludis. One half the plates and tubes were incubated aerobically at 35 C and the other half were incubated anaerobically in an atmosphere of 95% H_2 and 5% CO_2. On days 3 and 14 post inoculation, 1.0 ml of broth from each tube was inoculated onto an additional set of four agar plates. After 7 and 14 days of incubation, agar plates were observed microscopically for evidence of mycoplasma colonies. The agar surface was flooded with Dienes stain for visualization of any existing colonies. In addition, virus and control fluids were subcultured in mycoplasma-free WI-38 cells and tested for presence of mycoplasma by staining with Hoescht stain.

The unclarified vaccine and control fluids showed no evidence of mycoplasma contamination and the positive controls showed characteristic growth in the test media.

Bacteriological tests (21 CFR 610.12).

Unclarified vaccine and control fluids showed no growth in thioglycollate and trypticase soy broth when tested for 14 days as per 21 CFR 610.12.

TESTS PERFORMED ON VACCINE AFTER CLARIFICATION

Identity

Neutralization tests were used to verify the identity of virus contained in the DEN-4 vaccine. Hyperimmune mouse ascitic fluid prepared against DEN-4 strain H-241 (prototype) and a homologous rhesus monkey antiserum to the vaccine parent DEN-4 strain 341750 Carib were used in standard plaque reduction neutralization tests.

Breakthrough Neutralization

Table 2. Neutralization of DEN-4 plaques from a breakthrough neutralization test of the vaccine virus in LLC-MK_2 cells. Virus was neutralized with a 1:10 dilution of DEN 4 (H-241) antiserum.

Plaque picks	Log neutralization index
5	1.7
10-2	1.7
20-1	2.0
20-2	1.5
20-3	2.2
Mean:	1.8

Plaque reduction neutralization tests

Plaque reduction neutralization tests using dilutions of prototype and homologous antisera against approximately 100 pfu of vaccine virus and prototype virus was performed. Endpoint titers, i.e., the dilution of antiserum needed to reduce the plaque count by 50 percent or the log neutralization indices were not significantly different in any of the comparisons (Table 3).

Table 3. Neutralization titers of DEN-4 prototype H-241, 4328-S strains and vaccine 341750 antisera against H-241 and 341750 vaccine viruses in LLC-MK_2 cell culture monolayers..

Virus	Antibody	LNI	$PRNT_{50}$ Titer
DEN-4 vaccine	H-241	1.7	60
DEN-4 vaccine	4328-S	2.2	ND
DEN-4 vaccine	341750		220
DEN-4 H-241	H-241		760
DEN-4 H-241	341750		250
DEN-4328-S	4328-S		640
DEN-4328-S	341750		ND

FINAL CONTAINER TESTS

Potency Tests.

The vaccine pool was titrated in LLC-MK_2 cell cultures by plaque assay. The fianl container virus titer (fluid pool) was 1.7×10^5 pfu/ml. The final container lyophilized virus titer was 8.7×10^4 pfu/ml.

General Safety Tests.

Two adult mice weighing 15-20 g were inoculated with vaccine final product, 0.5 ml intraperitoneally.
Both animals survived for 21 days without signs of illness.

Sterility.

Twenty final containers of vaccine were rehydrated and tested according to Reg. 21 CFR 610.12.
No evidence of mycoplasma, other bacterial, or fungal contamination was observed.

Identity.
The vaccine contained dengue virus type 4 by neutralization test using reference antibody.

MEDIA AND PROTOCOLS

Growth medium for primary canine kidney cell cultures.

Basal medium (Eagles) with Hanks salts Catalog 420-1200 GIBCO Laboratories, Grand Island, New York., and Ten percent (10%) fetal bovine serum, certified free of mycoplasma and adventitious bovine agents was used for growth medium.
Minimum essential medium (Eagle) with Earles' salts catalog F-11 GIBCO Laboratories, Grand Island, New York wtih two percent (2%) fetal calf serum was used for maintenance medium.
Other supplements:
2 mM L-glutamine
50 mcg/ml neomycin sulfate
100 mcg/ml streptomycin sulfate
1.58 mg/ml sodium bicarbonate

Dengue virus, type 4 mouse ascitic fluid antibody.

A mouse hyperimmune ascitic fluid antibody to DEN-4 (H-241) prototype virus prepared at WRAIR 5 Sept. 1970 by the method of Brandt et al, American J. of Tropical Medicine and Hygiene 16:339, 1967; and a mouse hyperimmune ascitic fluid antibody to DEN-4 (H-241) virus prepared at Yale 24 Jan. 1966 by Dr. Robert Shope were used for the neutralication identity tests.
Monotypic serum from a Macaca irus monkey inoculated with a single dose of DEN-4 strain 4328-S virus and a rhesus monkey inoculated with two doses of homologous DEN-4 strain 341750 (parent) virus were also used.

Media for Mycoplasma Testing

Semi-solid media

Mycoplasma broth Base (BBL-11458)	3.0 g
Bacto agar (Difco 0140-01-0)	0.15 g
Distilled water	135 ml

Heat to melt agar and adjust pH to 7.8 for glucose medium or to 7.0 for arginine medium, and autoclave 15 minutes at 121 C. Cool to 50-55 C. Add sterile:

Yeast extract (25% aqueous solution) (BBL 11929)	20 ml
Horse serum (GIBCO 210-6270)	40 ml
Phenol red (GIBCO 870-1160) (0.1% solution)	1 ml
L-arginine HCl (42% solution) (Kodak 2475)	1 ml
or	
Glucose (Difco 0155-17) (50% solutions)	2 ml

Agar medium

Mycoplasma broth (above, without the agar and phenol red)	200 ml
Bacto agar (Difco 0140-01-0)	1.6 g

Dienes stain (MR0058)

Methylene blue (Baker Q475-3)	2.5 g
Azure II (Baker B649-2)	1.25 g
Maltose (Difco 0168-15-1)	10.0 g
Distilled water, qs to	100 ml

For use dilute 1:100 in distilled water.

Fluid thioglycollate medium: (Difco 0256-05-6)

Soybean casein digest medium (trypticase soy): (BBL 11768)

Hoechst DNA stain

Hoechst stain No. 33258 (Hoechst Pharmaceutical, Sommerville, N.J.) (Aldrich 86,140-5)	5.0 mg
Thimersol (merthiolate) (Aldrich E3,525-7) (1% solution)	10 ml

For use mix 0.5 ml of stock solution with 100 ml sterile distilled water.

Indicator cell system: Mycoplasma-free WI-38 cells maintained in the Department of Tropical Medicine tissue culture laboratory. For test, use one day cultures on LabTek slides inoculated with vaccine virus and M. pneumoniae and M. arthrididis (positive controls). Uninoculated wells serve as negative controls. After four days incubation at 37 C, the medium is removed from the slides and the cultures are fixed)without drying) in acetic acid: methanol (1:3) for 10 minutes. The slides are air dried and stained with Hoechst DNA stain for 30 minutes in the dark. The slides are rinsed three times with distilled water, blot dried, mounted in

medium consisting of:

22.2 ml 0.1M citric acid
27.8 ml 0.2M disodium phosphate
50 ml glycerol
adjust pH to 5.5

The slides are examined for extranuclear DNA at 1000X with a fluorescent microscope fitted with a BG-12 filter.

REVERSE TRANSCRIPTASE (RNA-DEPENDENT DNA POLYMERASE) ASSAY

The assay is based on that used by the American Type Culture Collection and published in the Tissue Association Manual, vol. 5, No. 3, 1979, pp. 1147-1150; and that described by Hoffman, Banapour and Levy (Virology 147:326-335, 1985).

To 10 ul of cell suspension preparation (supplied by WRAIR) on ice add the following freshly made and well vortexed mixture:

	Final concentration
2.5 ul 1.66 mg/ml dATP	50mM
2.5 ul 1M Tris-HCL pH 8.0	5mM
2.5 ul 0.1M Dithiothreitol (freshly made)	5mM
2.5 ul 0.1M $MgCl_2$	5mM
or	
2.5 ul 0.01M $MnCl_2$	0.5mM
7.5 ul 1M KCL	150mM
2.5 ul 1% Triton X-100	0.05%
2.5 ul 6.7mM glutathion (freshly made)	0.3mM
2.5 ul 10mM EGTA	0.5mM
2.5 ul 1mg/ml rAdT (primer)	50ug/ml
2.5 ul 0.5mg/ml BSA	25ug/ml
7.5 ul diethylcarbonate-treated distilled water	

Hold on ice for 5 minutes to solubilize the enzyme.

Add 10 ul (30uCi) [^{3}H]dTTP, 7.5umM final concentration. Place in a shaking water bath at 37 C for 60 minutes.

Add 10 ul transfer RNA (1 unit/ml) and mix well.

Stop the reaction with 2.0 ml cold 0.01M sodium pyrophosphate in 1N HCl, vortex well, and add 2.0 ml of cold 10% trichloroacetic acid (TCA).

Hold in an ice bath (4 C) for 20 minutes to allow precipitate to form.

Vortex well and filter through a 24 mm Whatman GFA glass filter prresoaked in 5% TCA and rinse 5 times with 15 ml (total 75 ml) of chilled 0.01M sodium pyrophosphate in 1N HCl and once with 2 ml cold 95% ethanol.

Allow filters to dry at room temperature and place in aquasol-2 (New England Nuclear) scintillation cocktail, vortex, and hold in dark for at least 3 hours before counting in a beta scintillation spectrometer.

The following controls are included in each assay:

Negative controls: 1) supernatant fluids from uninfected cell cultures, and 2) reaction mixture containing [^{3}H]dTTP only.

Positive controls: 1) a commercial preparation of reverse transcriptase adjusted to give approximately 10,000 dpm, and 2) supernatant fluids from 3 day cultures of HTLV-1 infected lymphocyte (H-1) cultures.

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